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Effects of currently used pesticides and their mixtures on the function of thyroid hormone and aryl hydrocarbon receptor in cell culture

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ABSTRACT

Evidence suggest that exposure to pesticides can interfere with the endocrine system by multiple mechanisms. The endocrine disrupting potential of currently used pesticides in Denmark was analyzed as single compounds and in an equimolar mixture of 5 selected pesticides. The pesticides were previously analyzed for effects on the function of estrogen and androgen receptors, the aromatase enzyme and steroidogenesis in vitro. In this study, the effect on thyroid hormone (TH) function and aryl hydrocarbon receptor (AhR) transactivity was assessed using GH3 cell proliferation assay (T-screen) and AhR responsive luciferase reporter gene bioassay, respectively. Thirteen pesticides were analyzed as follows: 2-methyl-4-chlorophenoxyacetic acid, terbuthylazine, iodosulfuron-methyl-sodium, mesosulfuron-methyl, metsulfuron-methyl, chlormequat chloride, bitertanol, propiconazole, prothioconazole, mancozeb and its metabolite ethylene thiourea, cypermethrin, tau-fluvalinate, and malathion (currently banned in DK).

In the T-screen, prothioconazole, malathion, tau-fluvalinate, cypermethrin, terbuthylazine and mancozeb significantly stimulated and bitertanol and propiconazole slightly reduced the GH3 cell proliferation.

In the presence of triiodothyronine (T3), prothioconazole, tau-fluvalinate, propiconazole, cypermethrin and bitertanol significantly antagonized the T3-induced GH3 cell proliferation. Eleven of the tested pesticides agonized the AhR function, and bitertanol and prothioconazole inhibited the basal AhR activity. Bitertanol, propiconazole, prothioconazole and cypermethrin antagonized the TCDD-induced AhR transactivation at the highest tested concentration.

The 5-component mixture had inducing effect but the combined effect could not be predicted due to the presence of bitertanol eliciting inhibitory effect. Upon removal of bitertanol from the mixture, the remaining four pesticides acted additively.

In conclusion, our data suggest that pesticides currently used in Denmark can interfere with TH signaling and AhR function in vitro and might have the potential to cause endocrine disruption.

Introduction

Pesticides are man-made and intentionally designed and released into the environment to provide crop protection, preservation of food and materials and prevention of vector-borne diseases. Due to their widespread use in agriculture, in and around residence, pesticide exposure is ubiquitous and continuing and poses a potential health risk. Exposure among the general population occurs primarily through the ingestion of food that contains low levels of pesticide residues or through inhalation and/or dermal exposure in home and in the environment. Humans are exposed to complex mixtures of pesticide residues. A recent report from Denmark found 2–10 different pesticide residues in 28% of analyzed fruit and vegetables from Danish and foreign origin (during 2004–2011) with generally higher frequency of pesticides in samples of foreign origin (Petersen et al., 2013). The European Food Safety Authority (EFSA), testing the pesticide residues on food samples from 29 European countries in 2011, could detect nearly 400 pesticides in measurable amounts in more than 600 food products. They found that 44.7% of the food samples (5660 samples) contained measurable residues, within the legally permitted levels (MRL, maximum residue levels) (EFSA, 2014) and 1.9% of the samples exceeded the MRL.

Pesticides represent a major group of endocrine disrupting chemicals that have the potential to affect the normal hormonal function by interfering with synthesis, secretion, transport, metabolism,

Abbreviations: AhR, Aryl hydrocarbon receptor; CA, Concentration addition; CV, Coefficient of variation; CYP450, Cytochrome P450; DAR, Draft Assessment Report; DMSO, Dimethyl sulfoxide; ETU, Ethylenethiourea (mancozeb metabolite); HAH, Halogenated aromatic hydrocarbon; LDH, Lactate dehydrogenase; MCPA, 2-Methyl-4-chlorophenoxyacetic acid; PAH, Polycyclic aromatic hydrocarbon; PE, Proliferative effect; REP, Relative potency; SC, Solvent control; SD, Standard deviation; T3, Triiodothyronine; T4, Thyroxin; TCDD, 2,3,7,8-Tetrachlorodibenzo-p-dioxin; TH, Thyroid hormone; TR, Thyroid hormone receptor; TSH, Thyroid stimulating hormone

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binding action, or elimination of natural hormones present in the body ((IPCS), I.P.o.C.S., 2002). Exposure to pesticides can disrupt the endocrine system and has been associated to adverse health effects such as cancers, neurodegenerative disorders and reproductive disorders (Mostafalou and Abdollahi, 2013).

Many of the persistent pesticides such as organochlorines have been replaced by less or non-persistent, contemporary pesticides such as organophosphates, carbamates and pyrethroids. Although environmentally non-persistent, the extensive use of pest control results in a continuously low exposure of the general population to the more widely used pesticides. Residues of the non-persistent pesticides and their metabolites have been measured in human maternal and umbilical cord sera, and urine (Fortin et al., 2008; Barr et al., 2010a; Castorina et al., 2010). Data from the U.S. National Health and Nutrition Examination Survey (NHANES; 1999–2002) show that 67% of pregnant women had detectable levels of the pyrethroid metabolite 3-phenoxybenzoic acid with the geometric mean of 0.321 μg/L (0.316 μg/g creatinine) (Castorina et al., 2010). Their data also indicated chronic exposure of pregnant women in the Salinas Valley, a largely agricultural area (CHAMACOS cohort), to several currently-used organophosphates and organochlorine pesticides (Castorina et al., 2010). Another study measuring contemporary pesticides in children (6–11 years old) in Spain reported similar urinary concentrations of organophosphate metabolites compared to US population (geometric means ranged from 0.47 to 3.36 μg/g creatinine) (Roca et al., 2014).

There are a number of reports on the endocrine disrupting effects of pesticides mediated via direct interaction with nuclear hormone receptors such as estrogen receptors (Bonefeld Jorgensen et al., 1997; Andersen et al., 2002; Grunfeld and Bonefeld-Jorgensen, 2004; Bonefeld-Jorgensen et al., 2005) the androgen receptor (Andersen et al., 2002; Vinggaard et al., 2002; Luccio-Camelo and Prins, 2011; Kjeldsen et al., 2013) and with the thyroid hormone receptors (TRs) (Ghisari and Bonefeld-Jorgensen, 2005; Du et al., 2010) as well as on the aryl hydrocarbon receptor (AhR) (Long et al., 2003; Takeuchi et al., 2008).

Thyroid hormones (THs) regulate a number of biological processes such as neuronal proliferation, cell migration and differentiation. Many of the genes involved in the processes of brain maturation have been identified as being regulated by THs through binding to TRs (Bernal, 2007). A broad range of environmental contaminants can interfere with TH signaling mechanism at several levels, including TH binding to TRs and thereby affect TH regulated gene expression (Zoeller, 2005; Boas et al., 2012). Disruption of the thyroid system and change in TH levels during the fetal and postnatal developmental periods is known to cause irreversible mental retardation and neurological deficits (Howdeshell, 2002; Morreale de Escobar et al., 2004).

Previously, we investigated the TH disrupting potencies of three currently used pesticides in Denmark (prochloraz, iprodione and chlorpyrifos) using the GH3 cell proliferation assay (T-screen) (Ghisari and Bonefeld-Jorgensen, 2005). Our results showed that the organophosphate insecticide, chlorpyrifos, could induce TH-like stimulation of the GH3 cell proliferation, whereas the imidazole fungicides, iprodione and prochloraz, inhibited the T3-induced proliferation of the cells. Later studies in rats showed that, prochloraz decreased the concentration of thyroxin (T4) and thyroid stimulating hormone (TSH) in serum of exposed rats (Vinggaard et al., 2002). Similarly, gestational and postnatal exposure to chlorpyrifos caused reduction of serum T4 in dams and F1 mice (De Angelis et al., 2009). Prenatal exposure to chlorpyrifos produces small significant reduction in brain T4 levels in rats, suggesting its TH disrupting potential in the developing brain (Slotkin et al., 2013).

In another in vitro study nine pyrethroids including cypermethrin were evaluated for potential endocrine disrupting activities via nuclear hormone receptors including TRs using receptor-mediated reporter gene assays in CV-1 cell line, and TR antagonistic effect was found for most of the tested compounds (Du et al., 2010). Rat studies have suggested that pyrethroid insecticides are involved in altered serum TH levels (Akhtar et al., 1996; Kaul et al., 1996; Giray et al., 2010).

Human studies on relationship between non-persistent pesticide exposure and effects on thyroid hormone system are limited. A Danish study evaluating the thyroid function in Danish greenhouse workers exposed to pesticides, reported increased serum TSH and small decrease in T4/T3 levels (Toft et al., 2006). In an US study, an inverse association between urinary biomarker of chlorpyrifos exposure and free T4 was reported in adult men from an infertility clinic (Meeker et al., 2006). A cross-sectional study found associations between hypothyroidism and use of organochlorine insecticides, the fungicides maneb, mancozeb and benomyl, and the herbicide paraquat in female spouses of private pesticide applicators in the Agricultural Health Study (Goldner et al., 2010).

Since there is a cross-talk between the signaling pathways such as those mediated by AhR and nuclear receptors ER, AR and TR (reviewed in (Ohtake et al., 2009)), ligands of AhR may indirectly modulate the function of nuclear receptors, and therefore it is important to characterize the AhR activity of more pesticides. Being a ligand-dependent transcription factor, the AhR mediates most of the toxic and biological effects of polycyclic and halogenated aromatic hydrocarbons (PAHs/HAHs) and was reported to be associated with decreased fertility, reproductive tract problems, oxidative stress, and cancer development (Hankinson, 1995; Gasiewicz et al., 2008). In addition, AhR plays a significant role in various physiological and developmental processes such as cell proliferation and differentiation, in liver, in ovary development and immune system homeostasis and in tumor development (Barouki et al., 2007).

Mechanistically, the AhR functions in a manner similar to that of the steroid hormone receptors and stimulates transcription of adjacent genes including *CYP1A1* and *CYP1B1* encoding two members of cytochrome P450 family (CYP450) (Hankinson, 1995; Whitlock, 1999). Beside the classical AhR ligands, AhR can be activated by numerous chemicals including several pesticides (Denison and Nagy, 2003; Long et al., 2003; Kruger et al., 2008). Previously, we reported that eight of 23 pesticides previously used in Denmark revealed AhR-mediated activity in human and rat hepatoma cell lines (Long et al., 2003). In addition, some other pesticides from the group of organophosphorus, carbamate and urea-type herbicides have been reported to act as AhR agonists in AhR-mediated reporter gene bioassay (Ledirac et al., 1997; Denison et al., 1998; Takeuchi et al., 2008).

The present study was part of a larger research project (HOPE), in which an initial screening of 13 currently used pesticides and one metabolite was performed, applying a battery of in vitro assays, including assays for effects on the estrogen receptor, the androgen receptor, aromatase enzyme (Kjeldsen et al., 2013), and steroidogenesis (Taxvig et al., 2013). The aim of this in vitro screening was to elucidate potential mechanisms of action as well as to determine the potency of the pesticides prior to in vivo testing. While current risk assessment approaches are predominantly based on individual pesticides, human exposure to these chemicals occurs predominantly to a complex mixture. Therefore, a 5-component mixture of active pesticides was included in this study, to reveal whether additivity applies. The mixture was subsequently analyzed for endocrine activity in vivo in pregnant rats in order to investigate the in vitro–in vivo correlations (Bossi et al., 2013; Taxvig et al., 2013).

The selection of the test compounds was based on a list of pesticides that were registered and approved for use in Denmark (except for malathion banned in 2008) as well as their endocrine disrupting potential, as described both in the open literature and in EU Draft Assessment Reports (DARs) received from the Danish Environmental Protection Agency. More specifically, the test compounds were selected on the basis of (i) application of pesticide, (ii) amount of pesticide (in kg) used in Denmark in 2006, (iii) acreage (in hectares) treated with